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THE PURPOSE AND SIGNIFICANCE OF ULTRATRACE ANALYSIS OF DIBENZO-P-DIOXINS: THE CONCEPT OF RISK. L. Brownlee* and B.R. Hollebone, Chemistry Department, Carleton University, Ottawa, Ontario, K1S 5B6.

INTRODUCTION

The ultimate goal of the toxicology disipline is to identify, define and respond to the health risks of chemical agents. The advent of chemicals in the environment is relatively recent, beginning with the industrialization of Europe. Since the second world war the release of chemicals has increased alarmingly, the benefits of these chemicals in short-term growth, medical advancements and control of the environment through pesticides and fertilizers overshadowing any long term negative effects. Only in the last 25 years have government agencies been forced to acknowledge the harmful effects of many industrial chemicals. The onus has been on these agencies to accumulate the data necessary to control the chemical industry.

Government resources, in both manpower and money have gone into environmental monitoring and health assessment studies in order to define the risk of chemicals already in the environment. Using this knowledge, effective legislation to control future chemical release into the environment has begun only in the last decade. Much work is left to be done.

The health risk of a chemical can be defined as follows:

TOTAL RISK = SUM OF ALL EXPOSURES X SUM OF ALL HAZARDS (i)

in which, Exposure has the units, Dose and Hazard has the units, Response/Dose. Inserting these units into the equation;

RISK = DOSE X RESPONSE / DOSE (ii)

which has the dimension of a % change in behaviour. This is a very broad equation when considered in terms of the total environment. The Toxicologist must sift through all the variables of this equation to identify the key exposure and resulting hazard to determine the ultimate risk to man.

Exposure:

Chemicals are released into the environment by three routes; by air through waste emission; on land through application; and into water via effluent waste. Much of the air and land pollution will precipitate or leach into the water system. From these primary routes, pollutants move into the human food supply through meat, produce and drinking water. Quantification of exposure is an analytical problem. Routine monitoring studies of

air, land and water identify problem areas and pinpoint sources of pollution. Residue studies of plants and animals determine the effect these chemicals have on the food chain. These data are used to predict the chemical exposure of man.

A great deal of research into the measurement of exposure has been done. Many research groups including our own are presenting "state of the art" methodologies at this conference that will aid toward better understanding of chemical exposure in Canada. Better analytical and monitoring techniques will allow better identification of populations that are "at risk" from environmental exposure.

Hazard:

Two fundamental problems of Risk Assessment are the determination of at what levels of environmental exposure a chemical becomes a risk and what the consequences of that risk are. This encompasses the hazard part of the risk equation.

The traditional approach to determining the hazard of a chemical is to study the toxic symptoms of laboratory animals at the medical level in response to a particular chemical dose and to try and correlate this dose to biochemical derangements.

In many studies the toxic medical responses of test animal to chemicals are the development of cancer or death. Detailed autopsies and extensive biochemical testing complement these studies to determine subcellular perturbations that could have resulted in the toxic medical symptoms. Linking these biochemical symptoms to the original chemical dose is a very labour intensive, complex and expensive mandate. Considering all the chemicals in the environment, a more streamlined and cost effective approach to screening potentially toxic chemicals is needed.

From the literature it is well understood that the hepatic Mixed Function Oxidase system is involved in drug tolerance and the activation of chemicals into carcinogens. Ten years ago these laboratories undertook to reverse the traditional approach to determine chemical hazard by studying the derangements of this system and how they relate to the ultimate toxic system.
Our objective was to understand the behaviour of the MFO system in response to <u>in vivo</u> and <u>in vitro</u> chemical exposure and with this information develop a screening procedure for exposure to toxic chemicals. This was done by identifying normal and abnormal biochemistry and correlating perturbations with the overall health of the animal.

MODEL OF RESPONSE OF DEFENSIVE ENZYME SYSTEMS

The Mixed Function Oxidase system, also known as Cytochrome

P-450, is a group of ubiquitous hemoproteins found in microorganisms, plants and animals, whose main purpose is to oxygenate lipophilic, non-nutrient compounds in order to eliminate them from the host (1). From a toxicology point of view, the hepatic microsomal P-450 found in birds and mammals is most interesting. As well as metabolizing certain endogenous compounds, hepatic P-450 detoxifies drugs and other xenobiotics. Most of the time this system works very efficiently to maintain biochemical homeostasis. When confronted with an excess of chemical, i.e. administration of a therapeutic drug, this system can function too efficiently, creating a tolerance and decreasing the effectiveness of the drug. In other cases this process may function in error and instead of detoxifying the chemical agent, the enzyme may convert the substrate into a mutagenic and/or carginogenic form. It is this characteristic that most interests toxicologists.

Cytochrome P-450 is a large globular octameric protein embedded into two dimensional lipid film called the endoplasimc reticulum (ER) of the cell. In hepatic ER, NADPH-Cytochrome P-450 reductase is also present. This enzyme system catalyzes electron transfer from NADPH to the cytochrome during the oxygen metabolizing process (2). In the traditional model (3), cytochrome P-450 is described as being a rosette surrounding the reductase enzyme. In a second model, the reductase moves like a ship through the P-450 molecules, simulating a sea of rocks. The stoichiometry of P-450 molecules is between 10:1 and 30:1 (3). In this model, the active site of P-450 is an iron protoporphyrin IX moiety that is located in a large, relatively open hydrophobic pocket in the surface of the protein (3) and the xenobiotic molecule is bound directly from the aqueous cytosol of the cell.

The inducibility of Cytochrome P-450:

In the absence of xenobiotics, Cytochrome P-450 in barrier and disposal tissues is at low levels. The introduction of substrates causes synthesis of new protein within minutes. This induction of the MFO system involves simultaneous synthesis of all other components and the proliferation of new endoplasmic reticulum (4). Cytochrome P-450 is in fact, the generalized name given to a group of isozymes embedded in this new protein. Traditionally, induction of P-450 is defined as an increase in enzyme concentration or an increase in the activity of a specific substrate. When the substrate concentration in the liver is reduced, the enzyme is catabolized back to basal levels. The stimulation of the Cytochrome P-450 system results specifically in measurable increases in enzyme activity, concentration of enzyme, proliferation of endoplasmic reticulum and liver weight. This inducibility of Cytochrome P-450 plays an important role in drug tolerance, which is traditionally exemplified by barbituates such as phenobarbitol and hexabarbitol. This was the

characteristic that first drew attention to the liver metabolizing system.

The general form of Cytochrome P-450 may in fact be a mixture of several isoforms present together. Indeed, specific chemicals will induce immunochemically distinct isozymes of P-450. There are now 8-11 distinct forms of induced Cytochrome P-450 isolated.

The best documented isozyme is induced by polyaromatic hydrocarbons (PAH's) found distinctly in the left lobe of the liver, and is commonly called Cytochrome P448 (5). This isozyme was first implicated in cancer research by Conney and his associates in the 1950's (4). They found that when rats were treated with PAH's such as benzpyrene (BP) and 3-methylcolanthrene (3-MC), the ability of the hepatic microsomes to hydroxylate BP increased up to 50 fold with little proliferation of ER or increase in P-450 concentration. As PAH's were known carcinogens i.e. found in smog, car exhaust and cigarette smoke, this unusually high activity was implicated in the carcinogenic mechanism.

In 1963 Fouts discovered that Cytochrome P-450 was very sensitive to chlorinated compounds when his animal room was fumigated with chlordane in the middle of a chronic feeding study (6). Although the original experiment was ruined, this discovery initiated a whole new field of Cytochrome P-450 chemistry. This work was extended to other chlorinated environmental compounds such as DDT, aldrin, dieldrin and Arochlor. In the early 1970's this work was extended to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a by-product of 2,4,5-Trichlorophenoxyacetic acid (2,4,5-T) production that was becoming very controversial due to industrial accidents and Agent Orange exposure in Vietnam.

THE EXPERIMENTAL APPROACH OF THESE LABORATORIES

Traditionally, the mechanism of the Cytochrome P-450 system has been studied using biochemical techniques. In early work, chemicals administered *in vivo* to the test animals were chosen for their known effects on the MFO system. Chemicals used in *in vitro* biochemical assays were chosen for their metabolite detection characteristics. In the last decade, many sophisticated biochemical techniques such as immunochemistry have advanced the field. Using this approach, some excellent work in the comparison of induction between species and in defining the induction of isozymes has resulted.

After the MFO system was found to respond to chlorinated compounds, environmental scientists became interested in using the enzyme as an environmental probe. Although some toxicological information using existing biochemical methods was possible, these methods were too qualitative to determine the

biological hazard of a chemical without artificially modifying the MFO system. These laboratories have studied the MFO system using a more toxicological approach by studying the direct response of the MFO system to chemicals selected for specific chemical properties. The experiments were designed to study the *in vivo* and *in vitro* toxicities of chemical probes on the behaviour of this defensive enzyme system found in the microsomal fraction of rat liver.

The Total Hepatic Induction (THI) index:

The first step in our approach was to quantitate the biochemical response of the MFO system to these chemical probes. The Cytochrome P-450 system is a very flexable system and is known to have an array of biological responses that it can use in order to adapt to a xenobiotic. The most evident basic responses of the P-450 system itself include an increase in biotransform enzyme activity, increase in enzyme concentration, a proliferation of endoplasmic reticulum and an enlargement of the liver (1). A quantitative system to monitor these responses would give an indication of the ability of the organism to cope with specific chemical stress and therefore give and indication of the "hazard" presented by that chemical. Using these responses, the Total Hepatic Induction (THI) index was developed to monitor the total liver microsomal response to inducer (7). This index was designed to quantitate, rather then replace detailed biochemical studies by providing a simplified picture of a complicated response.

This index was based on the assumption that the Mixed Function Oxidase (MFO) system was the rate limiting step in the metabolism and excretion of lipophilic xenobiotics in the body and that the steps preceding and following hydroxylation do not inhibit the process.

The total experimental index can be expressed as follows:

$$THI_{experimental} = \frac{\text{Enzyme activity}}{\text{[Cytochrome P-450]}} \times \frac{\text{[Cytochrome P-450]}}{\text{[Microsomal Protein]}} \times \frac{\text{[Microsomal Protein]}}{\text{Liver Weight}} \times \frac{\text{Liver Weight}}{\text{Animal weight}}$$

$$= \frac{\text{Enzyme Activity}}{\text{Animal Weight}} \qquad \text{(iii)}$$

Each component represents a change in state affected by the xenobiotic, hence, all are presented relative to controls. The dose response of each parameter is dependent upon the detoxification requirements and each parameter tends to compensate for any deficiency in response of another parameter. The THI index can be used to either provide a basis of comparing the total hepatic response of an individual to an xenobiotic or to compare responses between species to environmental contaminants.

This THI index has been used in our laboratories since 1982 on a routine basis. We have often found that a statistically significant difference from control of one parameter i.e. an increase in enzyme activity per P-450 concentration can be countered by an opposite change in a second parameter i.e. a decrease in the concentration of P-450 per unit protein. The final THI parameter was not statistically different from control animals. Statistically different THI indexes occurred when the response of an isozyme such as Cytochrome P-448 was evoked. This compensation mechanism is not normally seen when using typical biochemical techniques and suggests that the MFO system has a chemical response system that is more subtle then the well known isozyme response.

The MFO adaptation to C-H bond strength:

The second step in our toxicological approach was to examine the possible mechanisms of control at the active site of the enzyme system (8).

In most biochemical processes the enzymes are highly specialized to the geometry of one or a small number of substrates. For these particular compounds, the barrier to reaction is reduced by very specific control of the entropy of the reaction transition state ($\triangle S^*$). However, like all catalysts, enzymes may also assist the chemical change by providing sufficient energy to break old bonds or make new bonds, hence controlling the enthalpy of reaction ($\triangle H^*$). The sum of these two properties at any temperature is the free energy of activation of the reaction ($\triangle G^*$) where:

$$\triangle G^* = \triangle H^* - T\triangle S^* \qquad \text{(iv)}$$

In this regard, the MFO system is unusual in that the general form of Cytochrome P-450 has the ability to hydroxylate a wide range of geometrically unrelated xenobiotic substrates. The

number of identifiable Cytochrome P-450 isozymes is very limited in comparison with the number of known inducers. This lack of substrate specificity suggests that the MFO system has evolved a form of enthalpic control to determine the energy needed to insert activated oxygen into various substrates.

To test this concept, a three dimensional experiment was designed using the THI index to assess the relative importance of molecular shapes and bond strengths on the activity of the MFO active site (9). This experiment utilized chemicals chosen to meet specific chemical criteria of lipophilicity, structural simplicity, C-H bond strength and geometry.

Traditional experiments were designed to be two-dimensional. Animals were dosed with a chemical that "induced" the Cytochrome P-450 system. After a fixed time period to allow the animal to respond to the inducer it was killed and the prepared liver homogenate was assayed using substrates chosen for ease of analysis. Often there was no chemical similarity between the inducer and the substrate.

In our experiment, the inducers used <u>in vivo</u> and substrates for testing <u>in vitro</u> were chosen to be the same chemicals with C-H bond strengths that could be calculated from thermodymanic tables (10). Sprague-Dawley rats were tested with a sub-chronic 1/5 LD_{50} dose of each chemical. NADPH reduction by each microsomal preparation was measured in the presence of each chemical as a substrate under conditions for pseudo-first-order kinetics. The high background of NADPH reduction was removed by normalization with control microsomal activity. In this way, the response of the MFO system to a chemical (<u>in vivo</u> response) and its resulting ability to metabolize other substrates (<u>in vitro</u> response) can be independently compared to the bond strength of the chemical.

The null hypothesis (H_o,total) is that the MFO system will respond linearly to enthalpic requirement, that is, the C-H bond strength of the chosen chemicals both <u>in vitro</u> and <u>in vivo</u>. A non-linear response would suggest an entropic or shape related response mechanism. This hypothesis will factor into two two-dimensional experiments:

$$H_o, \underline{\text{total}} = (\Sigma H_o, \underline{\text{in vitro}}) + (\Sigma H_o, \underline{\text{in vivo}}) \qquad \text{(v)}$$

(H_o, <u>in vitro</u>) is defined as the adaptation of MFO to the bond strength of the inducer such that substrates with a lower C-H bond strength would be metabolized with more efficiency <u>in vitro</u> than the inducer as a substrate and substrates with a higher C-H bond strength with less efficiency. We have termed this null hypothesis as the <u>In Vitro</u> self-substrate test as it examines MFO ability to handle substrates from the perspective of the inducer. There will be a H_o or self-substrate test for each

inducer in the total experiment. Subtracting the self-substrate activity from other substrates activities for an inducer reduces the activity data to a series of positive, negative and zero numbers that, when examined in relationship with the substrate bond strength will characterize the Free Energy of Activation ($\Delta G_{r\rightarrow s}$, in vitro) of the MFO system's in vitro substrate interaction.

These data are given in figure 1. Data regression analysis of seven of the self-substrate tests suggest there is a linear relationship between the bond strength of the weakest C-H bond in the substrate to be metabolized and the free energy of activation induced by the animal. Those noted by asterisks were not included in the regression as they did not conform with the overall linearity of the experiment.

The second half of the total null hypothesis, (H_o, in vivo), or the In Vivo self-substrate test examines the experiment from the perspective of the substrate. An in vitro substrate with a known weakest C-H bond strength will be metabolized more efficiently by MFO adapted to an inducer with a stronger C-H bond strength than MFO adapted to the self-substrate and metabolized less efficiently by MFO adapted to inducers with a weaker C-H bond strength. A high $\Delta G_{r\rightarrow s}$ predicts high activation adaption of MFO to a strong C-H bond.

Data regression analysis in figures 2 indicates that there are distinct relationships between C-H bond strength and $\Delta G_{r\rightarrow s}$, in vivo of the ten substrates in this experiment that can be grouped according to the chemical properties of the weakest C-H bond.

In the first category are hexamethylethane (HME), cyclohexane (CY) and dimethylbutane (DMB), which have weakest C-H bonds of aliphatic primary, secondary and tertiary character respectively. As these substrates have no functional groups, they typify the baseline or "intended" behaviour of the MFO system. Most endogenous chemicals that are controlled by cytochrome P-450 are known to be aliphatic.

In the second category are 1,1,1-trichloroethane (TCE), lindane (LIN), chloroform (CHL), which are chlorinated aliphatic compounds; cyclohexene (CE), which is an unsaturated aliphatic compounds; and 124-trichlorobenzene (124) and 135-trichlorobenzene (135), which are chlorinated aromatic compounds. All of these chemicals contain weakest C-H bonds that have been influenced by the presence of other functional groups. These substrates have a much higher $\Delta G_{r\rightarrow s}$, in vivo than expected. The two data regression lines intersect at 101 kcal, which is the C-H bond strength of a hydrogen adjacent to an aliphatic double bond.

Benzene has aromatic C-H bonds which are stronger than any in aliphatic compounds and lie at the point of intersection of the two in vivo trends. It exhibits a much lower $\Delta G_{r\rightarrow s}$, in vivo than expected and does not fit into either linear relationship in vitro. All substrate activities, especially the self-substrate rate have decreased, apparently shifting the chemical to a chemical potential of MFO adapted to a weaker C-H bond strength. Benzene has been identified as a suicidal inducer (11) and a cancer initiator, and is metabolized to an epoxide. This compound is not the intended phenol. It is water insoluble and is not eliminated from the lipid into phase II of excretion. The epoxide instead can then destroy surrounding protein by free radical decomposition, resulting in a low observed activity in vitro. This apparent shift in bond strength position as a result of decreased activity in the in vivo self-substrate assay has been termed the Suicide Shift.

THE ASSESSMENT OF RISK

Defining the health risk of a chemical requires understanding of both the exposure to the chemical and the hazardous consequences of this exposure. Measurement of exposure is a problem of analytical chemistry. It requires development of convenient, reliable and sensitive methodologies that can be implemented in an extensive monitoring program. Proper measurement of exposure will identify those populations at risk.

The medical consequences or total hazard of the chemical requires an assessment and priorization of all undesirable biological changes. In the past, hazard has often been measured by the clinical presence of disease. Today, when exposure will result in the development of a disease such as cancer within several decades, the clinical definition of hazard is both imprecise and observed only after irreversible damage has occurred.

The answer to this is to develop an understanding of the interaction of the chemical with the early defense mechanisms of the body, because it is the breakdown of these subcellular systems that initiate the process of disease. As the first step in development of a science of chemical pathology, the development of a rapid screening method for chemicals would be possible. Potential problem chemicals could be identified and recommended for more detailed study. One such defense mechanism is the Mixed Function Oxidase system which is known to respond to and eliminate a wide array of chemicals. The measurable affects of chemical exposure can be observed within hours of administration. This versatility is very unusual as most enzyme systems are structure specific.

The THI index provides a mechanism to compare the total response at all levels of organization from whole body to subcellular effects of xenobiotics with control systems.

However, the most important component of this response is the adaptation of chemical potential at the active site and the behaviour of the xenobiotic at this active site. The observation of either intended or unintended behaviour here has the most potential as a predictor of chemical disease. The intended function of the MFO system is to hydroxylate hydrophobic chemicals so they can be eliminated from the body. When this behaviour occurs, the xenobiotic is removed, hence the risk of disease is eliminated.

The present experiment suggests that, as its first line of defense, the MFO system responds to the enthalpic properties of the invading chemical in both the inductive response and elimination process. This independence from the structural shape of the foreign compound is consistant with the versatility of MFO systems.

As the intricacy of living systems have been discovered, it is consistantly observed that the most conservative and efficient cellular processes have evolved. The response of the MFO system to the weakest C-H bond strength in a foreign chemical that needs to be disposed of can be recognized as a further example of this tendency. As cytochrome P-450 is a general enzyme system, all chemical structures, endogenous as well as exogenous that are present have the potential to be metabolized. The stronger the oxidation potential of the active site, the more the chemical structures are vulnerable to attack, since any bond that is weaker then the target C-H bond can be metabolized in this enthalpic system. Thus, while performing its "intended" behaviour, the MFO system must strike a balance between under response, where the chemical potential of the active site is too weak to be effective on the target compound which is also the self-substrate and over response, where essential substituents may be metabolized. Malfunction of the MFO system in either direction can lead to unintended influence on the surrounding tissue, which if not corrected could lead to the development of disease. A good example of over response and of accidental oxidation of other sites in strong C-H bond systems is Benzene (figure 2).

Benzene has a C-H bond which is stronger than any aliphatic equivalent and, as an inducer, will generate a high oxidation potential. The generated oxidation potential behaves as predicted in the <u>In Vitro</u> self-substrate test (figure 1). However, in the absence of an ortho/para or meta directing substituent in the ring, the aromatic C-C bond is more suseptible to attack than the target C-H bond, and unintended epoxide instead of an intended phenol will result. This will set off a subsequent chain reaction of "accidental" reactions. The epoxides are more hydrophobic than the corresponding phenol and therefore remain in the lipid and because they are chemically unstable will breakdown to give free radicals. The free radicals

propagate, destroying the surrounding protein, including cytochrome P-450. This reduces the observable substrate activity, especially for the self-substrate benzene itself. This results in a displacement on the <u>In Vivo</u> self-substrate test (figure 2) which is termed a suicide shift. The unpaired electrons generated by the free radicals are very soluble in lipid and very long lived, consequently they will attack lipid at double bonds. It is known that tissue high in unsaturated fats will develop cancer in the presence of radicals (12) and medically that benzene is a cancer initiator. This suggests a direct connection between the suicide shift generated by benzene and the strength of the cancer initiation capability. It is possible that the displacement of cyclohexene from the linear relationship with C-H bond strength in the <u>In Vitro</u> self-substrate test (figure 1) is also indicative of a suicide shift and therefore of a cancer initiator.

In the <u>In Vivo</u> self-substrate test (figure 2), there was also a group of chemicals containing functional groups that generated an oxidation potential greater than would be expected from the structural type of the calculated weakest C-H bond. As discussed earlier, this higher than necessary oxidation potential will increase the risk of generated free radicals and of the accidental oxidation of surrounding tissue or other xenobiotics. Although the self-substrate xenobiotics of this group will not destroy themselves as with benzene, these compounds can act as promotors of cancer by attack on less stable compounds such as unsaturated fats, generating free radicals through accidental behaviour.

In conclusion, the relationship between the MFO system and chemical C-H bond strength of the target xenobiotic provides a unique opportunity to study acute, subcellular effects of a chemical in a living system. As the analytical properties of this test is dependent upon relative response relationships, the reliability range of this assay will increase with further research. The relationship with C-H bond strength will also provide a theoretical tool for predicting biological response towards a new chemical. A chemical identified by this test as an initiator or promotor can be referred for further testing of its chemical pathology. A forth dimension of dose response can also be added to this assay. By varying the dose fed to the test animal, the critical concentration where the chemical emerges from the baseline response to behave as a promotor or initiator can be identified. This test system can also be used to identify non-toxic xenobiotics processed as intended that can be used to monitor for exposure or can be recommended for industrial or commercial uses.

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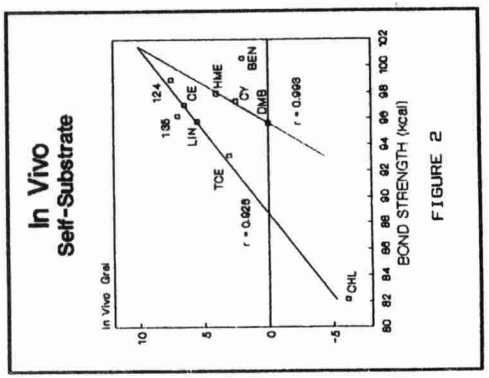

FIGURE 2

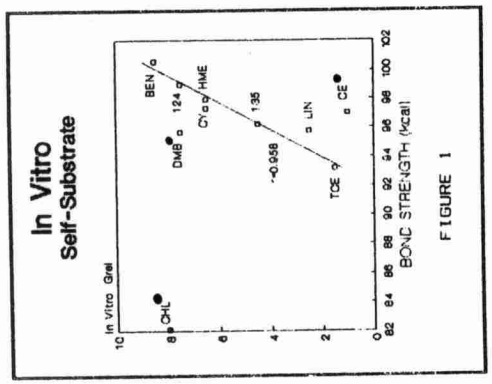

FIGURE 1

The In Vitro self-substrate test (figure 1) examines MFO ability to handle substrates from the perspective of the inducer.
The In Vivo self-substrate test examines the same data from the perspective of the substrate. Further details can be found in the text.
The substrates are: 1,1,1-Trichloroethane (TCE); Lindane (LIN); Cyclohexene (CE); Chloroform (CHL); 2,3-Dimethylbutane (DMB); Cyclohexane (CY); Hexamethylethane (HME); 1,2,4-Trichlorobenzene (124); 1,3,5-Trichlorobenzene (135) and Benzene (BEN).